Analysis of MLN4924 (Pevonedistat) as a Potential Therapeutic Agent in Malignant Melanoma

A Senior Honors Thesis

Presented in Fulfillment of the Requirements for Graduation with Honors Research Distinction in the Undergraduate Colleges of The Ohio State University

By

Gonzalo N. Olaverria Salavaggione

The Ohio State University
April 2017

Project Advisor:
Dr. William Carson III, Department of Surgery
Abstract
In this study, we evaluate MLN4924 (pevonedistat); a small but potent NEDD8 pathway inhibitor. The ubiquitin protease system (UPS) is a specialized protein complex that plays an important role in the ordered degradation of apoptotic stimuli within normal and malignant cells. The NEDD8 pathway is known to be a mechanism for the downstream activation of the UPS. Therefore, we hypothesized that MLN4924 treatment would induce apoptosis in human melanoma cells. A375 and Mel39 melanoma cell lines were treated with MLN4924 alone or in combination with interferon-alfa (IFN-α) – an immune boosting agent– or vemurafenib – a novel therapeutic used to treat patients with BRAF-mutant melanoma. Treatment with MLN4924 for 72 hours induced apoptosis in both A375 and Mel39 melanoma cells with IC_{50}s of 1200 nM and 143 nM respectively. Moreover, combination therapy of A375 cells with 10^4 Units/mL IFN-alfa and 1200 nM MLN4924 led to cell death levels of 78.2±3.7%, which is a significant synergistic increase compared to individual treatment by either therapeutic (p<0.005). Treatment of A375 cells with 1 μM vemurafenib had a notable effect on cell viability. However, when used in combination with MLN4924 there was an inhibitory effect on apoptosis. Results from MTS proliferation assays indicate MLN4924 does have anti-proliferation potential in melanoma cell lines, with or without the addition of IFN-alfa. A pre-treatment analysis also revealed MLN4924 led to the sensitization of A375 cells to vemurafenib treatment. Lastly, immunoblots of associate MLN4924-induced apoptosis to the cleavage of caspase-3, caspase-7, caspase-9 and PARP. These results demonstrate MLN4924 does have efficacy in treating advanced melanoma alone or in combination with IFN-alfa or vemurafenib.
Introduction

Melanoma is one of the deadliest forms of cancer today, with the average 10-year survival rate for stage 4 melanoma cases being 10-15% [1]. While melanoma composes about 1% of all skin cancer cases in the United States, it accounts for the majority of skin cancer related deaths. Incidence rates have steadily been increasing for the last 30 years, and in 2017 there will be an estimated 87,110 new melanoma cases, leading to an estimated 9,730 deaths [2]. While traditional treatments include surgical removal of the tumor, radiation therapy and chemotherapy, the most common form of therapy in advanced cases is immunotherapy. The mechanism by which the immune system functions involves an intricate system of checks and balances that is in place to prevent misguided attacks by immune cells on normal healthy cells. Unfortunately, malignant cancers (including melanoma) take advantage of these mechanisms by secreting cytokines that suppress the immune systems response, thus allowing the tumor cells to grow and proliferate [3]. Therefore, many immunotherapeutics focus on increasing the ability of the host lymphocytes to attack the malignant cells. Some of these therapeutics are immune checkpoint inhibitors such as antibodies specific for PD-1 and CTLA-4. Others are cytokines such as Interferon-alfa and interleukin-2 that boost the immune system’s sensitivity to stressors [4]. While these therapeutics have shown significant efficacy in fighting metastatic melanoma, detrimental side-effects are present [5]. Despite all available options and the great strides performed over the recent decades, immunotherapies and other treatments have demonstrated little success in eradicating metastatic melanoma [6].
About 50% of melanoma cases contain a mutation in the proto-oncogene \textit{BRAF}, which causes continuous activation of the mitogen activated protein kinase (MAPK) pathway, ultimately leading to actively proliferating malignant cells \cite{7}. To counteract continuous MAPK activation, the BRAF-inhibitor vemurafenib was approved in 2011 and had great initial success in containing disease progression. Unfortunately, resistance to vemurafenib along with an aggressive relapse of the cancer was observed in about 50% of cases after an average treatment of seven months. While the mechanism is not fully understood, it may involve the re-activation of the MAPK pathway \cite{8}.

The NEDD8 protein (81-amino acids in length) is the sole activator of the NEDD8 pathway; a complex mechanism that leads to the targeted degradation of apoptotic stimuli through utilization of the ubiquitin-protease system (UPS) \cite{9}. These apoptotic stimuli are often involved in arresting cell-cycle progression and preventing cells from proceeding through mitosis. The NEDD8 pathway begins with the conjugation of the NEDD8 protein to the NEDD8 activating enzymes (NAE). At this point, a cascade of trans-neddylation reactions occurs, transferring NEDD8 from the NAE-NEDD8 complex to one of multiple possible E2 complexes, and subsequently transferring it to a family of E3 ubiquitin ligases that are otherwise known as cullin-RING ligases (CRLs). A neddylated CRL can then conjugate a chain of ubiquitin molecules to a substrate (apoptotic stimulus), thereby targeting it for proteasomal degradation and thus allowing for cell growth and proliferation \cite{9}. Studies have shown that CRLs are responsible for ubiquinating an estimated 20% of proteins targeted for degradation through the UPS \cite{10}. Therefore, the NEDD8 pathway plays an important role in maintaining cell viability and growth in malignant cells.
Interferon-alfa (IFN-α) is a cytokine commonly used as an immune-enhancing therapeutic. It is utilized in treating a wide range of cancers. A previous study demonstrated that treatment of metastatic melanoma with IFN-alfa led to regression of the disease in about 10-15% of patients [11]. Furthermore, a recently published paper from our group demonstrated that combination treatment of IFN-alfa with novel UPS inhibitor ixazomib (MLN2238) in melanoma enhanced levels of apoptosis by approximately 11% [12]. Another study performed by our group showed that IFN-alfa can act synergistically with the proteasome inhibitor bortezomib through the activation of caspases and by interacting with Fas and FADD proteins. Additionally, reduced melanoma tumor growth has been seen in murine models with combination treatment of IFN-alfa and bortezomib [13].

The novel NEDD8 activating enzyme (NAE) inhibitor MLN4924 (pevonedistat) is a potential new therapeutic approach to treat melanoma. Produced by Millennium Pharmaceuticals, MLN4924 works by hindering the NEDD-8 pathway. Specifically, it inhibits the formation of the NAE-NEDD8 complex, thereby preventing the cascade of reactions that will lead to the degradation of apoptotic stimuli via the recruitment of the UPS. This results in the persistence of apoptotic stimuli within the cell, ultimately leading to cell death, as seen in a study that found MLN4924 was able to suppress proliferation and migration of urothelial carcinoma [14]. While still preliminary, many ongoing studies have found MLN4924 to have efficacy as a therapeutic agent in a broad range of cancers. MLN4924’s effects on patients with malignant melanoma remain to be seen, yet we hypothesize that it will suppress tumor cell growth and proliferation, while inducing significant levels of apoptosis in vitro. We also hypothesize that MLN4924 will work...
synergistically with other common melanoma therapeutics, such as IFN-alfa and vemurafenib, to further increase its cytotoxic potential.
Material and Methods:

Materials: The A375 human melanoma cell line was purchased from the American Type Culture Collection (ATCC Manassas, Virginia). The A375 vemurafenib-resistant cell line was created in Dr. Carson’s laboratory over a period of time by exposing wild-type A375 to increasing doses of vemurafenib. The human metastatic melanoma cell line Mel39 was a gift from Soldano Ferrone (Harvard Medical School, Boston, MA) and cultured in DMEM media supplemented 10% FBS, L-glutamine, and penicillin/streptomycin. Melanoma cell line M308 was kindly provided by Dr. Antoni Ribas (University of California, Los Angeles) and was maintained in RPMI-1640 supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. MLN4924 (Velcade, PS-341) was obtained from Millennium Pharmaceuticals, Inc. (Cambridge, MA) and recombinant human IFN-alfa was obtained from Schering-Plough, Inc (Kenilworth, NJ). Vemurafenib was obtained from Selleckchem.

Annexin V-propidium iodine staining: Apoptosis-induced phosphatidylserine exposure and cell membrane integrity was measured in tumor cells by flow cytometric analysis using APC-Annexin V and PE-propidium iodide (PI; BD Pharmingen, San Jose, CA), as previously described [18]. Each analysis was performed using 10,000 – 30,000 events and provides the percentage of the cell population that is viable or apoptotic [18]. The externalization of phosphatidylserine (PS) residues on the cell membrane occurs first during apoptosis. Annexin V has a high affinity for PS and monitors it once PS transfers from the inner membrane to the outer membrane. Propidium iodide is not able to penetrate viable cell membranes. Once the integrity of the cell membrane begins to degrade during apoptosis, propidium iodide can then penetrate the cell. The combination
of these two agents together provides a useful assay to determine whether a cell population is viable, apoptotic, or necrotic [18]. The full method is described in Appendix A.

**Immunoblot analysis:** Immunoblots were prepared to probe with antibodies specific for caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, caspase-9, poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Danvers, MA), or β-actin (Sigma, St. Louis, MO). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, immune complexes were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Waltham, MA).

**Proliferation assays:** The proliferation of melanoma cells was measured as absorbance at 490 nm using the Cell Titer 96 Aqueous One Solution Cell Proliferation MTS Assay (Promega). All assays were performed in triplicate. Cells were also stained with trypan blue (Life Technologies, Carlsbad, CA) and live/dead cell counting was used to confirm proliferation assay results.
Results:

I. Treatment of BRAF (V600E) mutant human melanoma cell lines with MLN4924 led to apoptotic cell death. MLN4924’s potential to induce apoptosis was explored in the BRAF V600E mutant cell lines A375 and Mel39. Cell death was measured with Annexin V/PI staining with a treatment length of 72 hours as smaller time spans showed low levels of apoptosis. Since only sparse data was available prior to the start of this project, it was necessary to perform a titration over a wide range of concentrations to deduce the IC$_{50}$ of MLN4924 in each cell line. Figure 1 shows a full titration with A375 cells, starting with an untreated (negative) control and increasing concentrations of MLN4924 from 25 nM to 3200 nM. Figure 2A displays a comparison of an untreated control exhibiting 9.58% cell death vs. the calculated IC$_{50}$ of 1200 nM resulting in 54.1% cell death. Figure 2B shows a graphical analysis of the titration in A375 cells. Figure 3 shows titration of MLN4924 in Mel39 cells. Part A is a graphical analysis of the titration and the calculated IC$_{50}$ concentration of MLN4924 in Mel39 was found to be 143 nM. Part B compares an untreated control (14.9% cell death) with a treatment concentration of 150 nM (52.2% cell death).
Figure 1. MLN4924 induces apoptosis with 72-hours of treatment in the BRAF mutant (A375) cell line. A375 BRAF mutant (V600E) human melanoma cells were plated on a 6-well plate at a density of 200,000 cells/well and were either untreated or treated with varying concentrations of MLN4924 starting at 25nM and up to 3200 nM. Cells were incubated with drug for 72 hours to obtain optimal apoptosis levels. After incubation, flow cytometry analysis utilizing Annexin V and Propodium Iodide (PI) antibodies were utilized to determine apoptosis percentages.
Figure 2. MLN4924 induced apoptosis in A375 BRAF (V600E) mutant human melanoma cell line with an IC$_{50}$ of 1200 nM. A375 BRAF mutant (V600E) human melanoma cells were plated on a 6-well plate at a total amount of 200,000 cells/well. Cells were untreated or treated for 72 hours with increasing doses of MLN4924. The cells were subjected to flow cytometry analysis via use of Annexin V/PI antibodies to determine cell death percentages post-incubation. A) Displays a graphical analysis of A375 cell line titrations with MLN4924 at 72 hours. From these results, the IC$_{50}$ for MLN4924 in A375 cells was determined to be 1200 nM. B) IC$_{50}$ concentration (1200 nM) was tested to ensure accuracy of data. Figure shows a comparison between an untreated control and the IC$_{50}$ concentration. Apoptosis levels were 9.48±0.7% and 54.1±1.7% respectively.
Figure 3. MLN4924 induced apoptosis in Mel39 BRAF (V600E) mutant human melanoma cell line with an IC₅₀ of 143nM. Mel39 BRAF mutant (V600E) human melanoma cells were plated on a 6-well plate at a final amount of 200,000 cells/well. Cells were untreated or treated for 72 hours with increasing doses of MLN4924 ranging from 50 nM to 400 nM. The cells were subjected to flow cytometry analysis via use of Annexin V/PI antibodies to determine cell death percentages. A) Displays a graphical analysis of Mel39 cells titrations with MLN4924 at 72 hours. From these results, the IC₅₀ for MLN4924 in Mel39 cells was determined to be 143 nM. B) Comparison of apoptosis percentages between an untreated control and a near-IC₅₀ concentration (150 nM). Apoptosis levels were 14.6±0.6% and 53.5±1.7% respectively.
II. Combination treatment of A375 BRAF (V600E) mutant human melanoma cell line with MLN4924 and IFN-alfa enhances therapeutic cytotoxicity. To assess the capabilities of MLN4924 to act synergistically with other therapeutics, A375 cells were treated for 72 hours with varying concentrations of MLN4924 (0 nM, 100 nM, 1200 nM or 2000 nM), IFN-alfa (1×10⁴ U/ml), vemurafenib (1 µM), or with MLN4924 in combination with IFN-alfa or vemurafenib. All therapeutics were added simultaneously and apoptosis levels were measured with Annexin V/PI flow cytometry staining. The combination of MLN4924 with IFN-alfa led to a significant additive level of apoptosis (78.2±3.7%) in A375 cells compared to MLN4924 treatment alone (50.7±1.0%) (Figure 4B) (p<0.005). However, the combination of MLN4924 and vemurafenib seems to not enhance apoptosis in A375 cells, as apoptosis levels were lower with the combination of vemurafenib and MLN4924 compared to individual MLN4924 treatment at higher doses (1200 nM & 2000 nM). Regardless, treatment with a low concentration of MLN4924 (100 nM) in combination with vemurafenib resulted in 40.7±0.7% cell death compared to 34.0±2.7% cell death of individual MLN4924 treatment (Figure 4B).
Figure 4. Combination treatment of A375 BRAF (V600E) mutant human melanoma cell line IFN-alfa led to additive apoptosis levels. A) Mutant BRAF (V600E) A375 human melanoma cells were treated for 72 hours with 1200 nM MLN4924, $10^4$ Units/mL IFN-α or 1 µM vemurafenib. Apoptosis levels were assessed by Annexin V/PI staining. B) Comparison of apoptosis levels between MLN4924 concentrations of 0 nM, 100 nM, 1200 nM and 2000 nM alone or in combination with $10^4$ U/mL IFN-α or with 1 µM vemurafenib. Treatment length was 72 hours and results indicate a significant additive increase in cell death with MLN4924 (1200 nM) and IFN-α compared to lone MLN4924 treatment (***p<0.005). Combination of MLN4924 and vemurafenib displayed a non-significant inhibitory effect as cell death levels dropped with higher MLN4924 concentrations compared to lone MLN4924 treatment.
III. Pre-treatment of the A375 BRAF (V600E) mutant human melanoma cell line with MLN4924 led to cell sensitization to vemurafenib treatment. It was previously shown that combination treatment of MLN4924 and vemurafenib in A375 cells led to lower levels of apoptosis compared to individual MLN4924 treatment. However, a recent publication by Wand et al. has shown that significant levels of NEDD8 inhibition was present within 6-24 hours after initial treatment with MLN4924 [15]. We thus explored the potential of MLN4924 to serve as a sensitizer of A375 BRAF (V600E) mutant melanoma cells to the FDA-approved BRAF inhibitor vemurafenib. Cells were treated with 1200 nM MLNL4924 for either the first 24 or the total 72 hours. Vemurafenib treatment (5 µM) was initiated at the 24-hour mark for a total treatment of 48 hours. For some of the treatments, the media containing MLN4924 was removed and replaced after the first 24 hours to prevent the mixing of MLN4924 with vemurafenib (as we previously observed an inhibitory effect). In figure 5, R indicates the media was removed and replaced at the 24-hours mark prior to the addition of vemurafenib and NR indicates the media was not removed and replaced. Cell death levels were measured with Annexin/PI flow cytometry. Figure 5 compares the cell death percentages between all groups, with the combination ‘R’ treatment yielding the highest cell death percentage (63.4±2.3%). This significant finding (p<0.05) may be indicative that pre-treatment of A375 BRAF mutant cells with MLN4924 led to sensitization to vemurafenib treatment.
Figure 5. Pre-treatment of A375 *BRAF (V600E)* mutant human melanoma cell line with MLN4924 led to cell sensitization to vemurafenib treatment. Mutant BRAF (V600E) A375 human melanoma cells were incubated for a total 72 hours. Treatment with MLN4924 (1200 nM) was for either the first 24 hours (R) or the entire 72 hours (NR). Treatment with vemurafenib (1 µM) was started at the 24-hour mark and maintained through the remaining 48 hours. Results indicate no significant variation in cell death when comparing MLN4924 NR (52.5±0.6%), MLN4924 R (57.1±8.2%) and MLN4924+vemurafenib NR (55.9±1.0%) treatments. MLN4924+vemurafenib R treatment resulted in an apoptosis percentage of 63.4±2.4%, which is a significant increase compared MLN4924 NR treatment (*p<0.05). However, comparison of MLN4924+vemurafenib NR vs MLN4924+vemurafenib R treatments showed no significant increase in cell death percentages.
IV. MLN4924 treatment alone and in combination with IFN-alfa resulted in reduced cell proliferation in BRAF mutant human melanoma tumor cells as measured by MTS assays. Cell proliferation was assessed using the MTS assay by measuring absorbance (O.D. 470 nm) to quantify the anti-proliferation potential of MLN4924. A375 BRAF mutant melanoma tumor cells were treated with various concentrations of MLN4924 to create a cell proliferation titration curve (Figure 6A). Results indicate a decrease in cell proliferation with increasing concentration of MLN4924 treatment with an IC$_{50}$ of 408 nM. Combination treatments with 1200 nM MLN4924 and IFN-alfa ($1 \times 10^4$ U/mL) or vemurafenib (1 µM) on A375 melanoma cells were also conducted to assess their effects on cell proliferation while comparing combination treatment effectiveness to single therapeutic treatment (Figure 6B). Titration of MLN4924 (Figure 6C) along with the combination treatment with IFN-alfa or vemurafenib (Figure 6D) was repeated in Mel39 BRAF mutant melanoma tumor cells. The IC$_{50}$ of MLN4924 in Mel39 cells was 142 nM. Regarding the titration with various concentrations of MLN4924, proliferation for both A375 and Mel39 cells was greatly inhibited, with Mel39 cells being more sensitive to treatment compared to A375 cells (as similarly seen in the cytotoxicity analysis from section I). Regarding the combination treatment, both the A375 and Mel39 cells were treated with 1200 nM MLN4924 for simplicity reasons when comparing the results. Alike the apoptosis analysis, MLN4924 functioned additively when combined with IFN-alfa, yet there was an inhibitory effect when combined with vemurafenib. Overall, MLN4924 demonstrates efficacy in inhibiting melanoma tumor cell proliferation and this effect is enhanced with the addition of IFN-alfa.
A

% Cell Proliferation (O.D. 490nm) vs MLN4924 Concentration (nM)

B

% Cell Proliferation (O.D. 490nm) for different treatments:
- Untreated
- MLN4924 1200 nM
- IFN-α 10^4 U/ml
- MLN+IFN
- Vem 1uM
- MLN+Vem

Significance levels:
- *** indicates a significant difference
C

% Cell Proliferation (O.D. 490nm)

MLN4924 Concentration (nM)

D

% Cell Proliferation (O.D. 490nm)

Untreated  MLN4924 1200 nM  IFN-α 10^4 U/ml  MLN+IFN  Vem 1uM  MLN+Vem

* *
Figure 6. MLN4924 combination treatment with IFN-alfa but not vemurafenib resulted in inhibited cell proliferation in BRAF mutant human melanoma cells as measured by MTS assay (O.D. 490 nm). A) Human melanoma A375 tumor cells were plated at a final amount of ~5000 cells/well in a 96 well plate and treated with various concentrations of MLN4924 for 72 hours. Following incubation, 20 ul of the MTS reagent was added to each well and the plate was incubated for an additional 30 minutes. The plate was read in a plate reader at 490 nm to measure cell proliferation and the proliferation IC₅₀ was determined to be 408 nM. B) The same procedure was repeated with A375 cells using 1200 nM MLN4924 with or without IFN-alfa (10⁴ U/mL) or vemurafenib (1 µM) for the A375 BRAF mutant cell line. Combination treatment resulted in a significant reduction in tumor cell proliferation with IFN-alfa but a significant inhibitory effect was observed with vemurafenib. C) Procedure from section A was repeated with Mel39 cells. The IC₅₀ value of Mel39 for proliferation was determined to be 142 nM. D) The combination treatment was repeated in Mel39 cells using 1200 nM MLN4924 with or without IFN-alfa (10⁴ U/mL) or vemurafenib (1 µM). Alike the results from section B, combination treatment resulted in a significant reduction in tumor cell proliferation with IFN-alfa and a significant inhibitory effect with vemurafenib (*p<0.05, **p<0.005, ***p<0.0005).
V. Treatment of A375 Vem and M308 vemurafenib-resistant human melanoma tumor cells with MLN4924 led to induced apoptosis. A375 vemurafenib-resistant (A375 Vem) cells were created in an in vitro setting from A375 BRAF (V600E) mutant human melanoma cells by a slow and constant exposure to vemurafenib. M308 vemurafenib-resistant cells were isolated from a tumor of a patient who had acquired vemurafenib resistance during clinical treatment. Cells were treated with various concentrations of MLN4924 (0 nM, 100 nM, 1200 nM & 2000 nM) and incubated for 72 hours. Apoptosis levels were measured with Annexin V/PI flow cytometry staining. Figure 7 compares the cell death percentages of wild-type A375, A375 Vem and M308 vemurafenib-resistant melanoma tumor cells. A significant increase in susceptibility to MLN4924 was seen in the A375 Vem cell line where vemurafenib resistance was induced in vitro, but the opposite effect was observed when vemurafenib resistance was induced in vivo. Unfortunately, the mechanism for the acquisition of vemurafenib resistance is still unknown, though studies suggest it may be due to the reactivation of the MAPK or the PI3K/AKT pathways [18]. These findings ultimately do not allow us to definitively conclude whether vemurafenib resistance leads to susceptibility to MLN4924 treatment, yet it does indicate that MLN4924 may have efficacy in treating vemurafenib-resistant melanoma tumor cells.
Figure 7. Treatment of A375 and M308 vemurafenib-resistant human melanoma tumor cells with MLN4924 led to apoptotic cell death. Wild-type A375 BRAF mutant (V600E) human melanoma tumor cells were plated on a 6-well plate at a density of 200,000 cells/well and were treated for 72 hours with increasing doses of MLN4924 (0 nM, 100 nM, 1200 nM, 2000 nM). The same procedure was repeated for A375 (in vitro-acquired resistance) and M308 (in vivo acquired resistance) vemurafenib-resistant human melanoma tumor cells. Apoptosis levels were obtained through flow cytometry analysis via use of Annexin V/PI antibodies. Results demonstrate a significant increase in cell death percentage when comparing wild-type A375 cells and vemurafenib-resistant A375 cells. However, M308 vemurafenib-resistant cells demonstrated a significant lower response to MLN4924 treatment (*p<0.05, **p<0.005, ***p<0.0005).
VI. Immunoblot analysis displays downstream mechanisms for MLN4924 apoptosis induction. Caspases are a family of protease enzymes that are involved in the downstream execution of inducing apoptosis. Treatment of A375 BRAF (V600E) mutant human melanoma cells with MLN4924 for 72 hours enhanced the cleavage of caspases-3 but not caspase-7, caspase-9, or PARP.

**Figure 8:** MLN4924 treatment induces cleavage of apoptosis factor. A375 melanoma cells were treated with 0 nm, 1200 nM or 600 nM MLN4924. All treatments with MNL4924 resulted in increased cleavage of caspase-3 but not caspase-7, caspase-9 or PARP, which are all key proteins in the downstream induction of apoptosis. B-actin was used as a control for protein concentration.
Discussion:

MLN4924 (pevonedistat) is a novel NEDD8 activating enzyme (NAE) inhibitor that ultimately leads to the inhibition of the ubiquitin protease system (UPS), which is responsible for the targeted degradation of apoptotic stimuli [14]. In this study, we evaluated the efficacy of MLN4924 as a potential therapeutic agent in melanoma tumor cell lines. We hypothesized MLN4924 would stimulate apoptosis in human melanoma cells and that it would work synergistically when utilized in combination with the immune stimulant IFN-alfa or the BRAF-inhibitor vemurafenib. Our results demonstrate that MLN4924 does induce significant levels of apoptosis and reduced proliferation in BRAF mutant A375 and Mel39 melanoma tumor cell lines. Additionally, MLN4924 does demonstrate some potential for use in the treatment of melanoma tumor cells that have acquired to resistance to vemurafenib.

Combination therapy with MLN4924 and IFN-alfa resulted in an apparent synergistic increase in apoptosis levels for A375 BRAF mutant cell line. This apparent synergistic effect was also observed in the ability of combination treatment to reduce tumor cell proliferation in both BRAF mutant A375 and Mel39 melanoma cell lines. On the other hand, an inhibitory effect was observed when utilizing combination therapy of vemurafenib with increasing doses of MLN4924 in A375 BRAF mutant melanoma cells for both apoptosis levels and tumor cell proliferation rates. Nevertheless, MLN4924 demonstrated an ability to act as a sensitizing agent on to vemurafenib treatment in A375 cells when MLN4924 was removed from the cells prior to treatment with vemurafenib.

Our immunoblot analysis demonstrates that MLN4924-induced apoptosis was associated with the cleavage of caspase-3 but not caspase-7, caspase-9 or cleavage of
PARP. These results, apart from caspase-3, contradict our original predictions, as our untreated sample should have expressed the lowest amount of cleavage. However, other publications’ immunoblots show the results we expected for caspase-3, caspase-7, caspase-9 and PARP [24] [26]. The difference between our assay and theirs is these groups treated their cells for 48 hours, while in our study we treated them for 72 hours. This may explain the high levels of cleavage observed in our untreated sample as at the 72-hour mark the untreated A375 cells are close to being over-confluent. Because of this, it can lead the cells to begin undergoing apoptosis as there is no surface available for them to adhere and the essential nutrients present in the media have mostly been consumed. We intend to repeat our immunoblot analysis at a 48-hour treatment length to insure our untreated cells do not outgrow their resources and become apoptotic. Nevertheless, when comparing our 600 nM and 1200 nM MLN4924 treatments, we do observe an increase in the cleavage of all the caspases and PARP with increasing MLN4924 concentration.

Caspase-3 and caspase-7 are known as ‘executioner caspases’, meaning they are directly responsible for inducing apoptosis. Caspase-9 on the other hand is known as an intrinsic activator-caspase that is responsible for the activation of executioner caspases [21]. One group has presented a model mechanism for the activation of caspase-9 that begins with the inhibition of the NEDD8 pathway due to MLN4924, leading to the inhibition of the ubiquitin protease system which prevents the degradation of pro-apoptotic p21, p27, Noxa and Bik factors [24]. Since MLN4924 treatment stimulates the activation of caspase-9, this is indicative that MLN4924 induces apoptosis through an intrinsic (intracellular) pathway that is initiated by the inhibition of
the NEDD8 pathway. In regards to PARP, studies performed by other groups have verified cleavage of PARP to be a direct downstream result of the activation of caspase-3 and caspase-7 [22].

While our immunoblot results indicate an intrinsic mechanism by which apoptosis is induced, another study performed in human cervical carcinoma cells revealed apoptosis was induced via activation of caspase-8 as well [26]. This is interesting as caspase-8 is activated by an extrinsic pathway, meaning an extracellular ligand binds to a death receptor outside of the cell membrane leading to cleavage of caspase-8, which then cleaves executioner caspase-3, caspase-6 and caspase-7 thereby inducing apoptosis [21]. Since MLN4924 is a NEDD8 pathway inhibitor (an intracellular pathway), it implies its effects should be present within the cell and thus lead to an intrinsic induction of apoptosis (via caspase-9) as seen from our results. However, activation of caspase-8 is indicative of an extrinsic induction of apoptosis, meaning MLN4924 might have a secondary method to directly or indirectly induce apoptosis.

Aside from apoptosis stimulation, a study found that MLN4924 treatment in lymphoma cells led to the upregulation of p27 (cyclin-dependent kinase inhibitor) and p21 (tumor suppressor protein), both of which play roles in inhibiting cell cycle progression [24]. This may explain the reduction in proliferation observed in our A375 and Mel39 BRAF mutant melanoma cells.

In terms of MLN4924 efficacy against vemurafenib-resistant tumor cells, ongoing research from our group along with another published study have pointed to the mutation and upregulation of the NRAS gene as a mechanism for the acquisition of vemurafenib resistance by malignant cells [27]. While this mechanism is not fully understood yet, there
is no link present between the NEDD8 pathway and NRAS gene expression that would imply malignant cells are more susceptible to MLN4924 treatment compared to non-vemurafenib resistant malignant cells.

MLN4924 is a novel therapeutic agent and multiple other publications have assessed MLN4924’s efficacy as a therapeutic in malignant melanoma. A phase I clinical trial conducted on metastatic melanoma has stated that MLN4924 led to a partial response in one patient and stable disease in 15 others (4 lasting ≥ 6.5 months). It also determined the maximum tolerated dose to be 209 mg/m² [19]. Another study has shown that pre-clinical treatment of various melanoma cell lines with MLN4924 led to reduced cell viability and proliferation with an IC₅₀ <0.3 µM [20]. While our study has found similar results in assessing the potential of MLN4924 as a cytotoxic agent, we are the first to find that MLN4924 can work synergistically with IFN-alfa as well as serve as a sensitizer to vemurafenib treatment. Additionally, we are the first to observe that MLN4924 has efficacy in treating vemurafenib-resistant melanoma tumor cells.

MLN4924 has shown potential in targeting other forms of cancers as well. One group found MLN4924 to be highly effective against human lymphoma cells (in vitro) compared to the classical proteasome inhibitor MG132. Interestingly, immunoblots showed the inhibition of the anti-apoptotic Bcl-2 and Mcl-1 protein families [24]. Another study performed in human urothelial carcinoma found MLN4924 to induce apoptosis and inhibit tumor cell proliferation in vitro. Studies in xenograph mouse models found dose-dependent cytotoxic effects, along with anti-proliferation, anti-migration and anti-invasion effects when mice were treated with MLN4924 [25].
Given our results and the published literature, MLN4924 is an exciting novel therapeutic agent that has shown efficacy in treating various forms of malignant cancers both in vitro and in vivo. MLN4924 has also been shown to have high specificity as well as a well-tolerated toxicity in mouse models and thus many clinical trials are currently ongoing [25]. Our group will continue to assess MLN4924’s potential as a melanoma therapeutic by conducting cytotoxicity and proliferation assays on MeWo BRAF wild-type melanoma tumor cells in vitro, along with utilizing mouse models to assess MLN4924’s efficacy in an in vivo setting.

Appendices:

Appendix A
Annexin/PI Protocol:

1. Isolate cells from a flask and transfer to a 15 mL conical tube.
2. Centrifuge the cells at 1700 RPM for 7 minutes.
3. Resuspend pellet in 5mL of complete media, and count cells using a 5uL aliquot into 95 uL of trypan blue solution.
4. Count the cells and determine volume required to obtain 200,000 cells.
5. Prepare and label your 6 well plates (Cellstar Product #657160). You do not want to add more than 200,000 cells per well, and want 100,000 cells per flow tube. For your positive and negative controls, you want to have an unstained flow tube, PI only stained flow tube, Annexin V only stained flow tube, and a Annexin V and PI stained flow tube. You also want to have these four flow tubes for each stimulus you have in order to be able to set your quadrants. For example, if you will have 4 flow tubes for a sample, you will have to have 2 wells for that condition and will then combine the cells after incubation.
6. Add 200,000 cells (volume will vary depending on cell concentration) per well in a 6 well plate.
7. Then add media to each well to reach a final volume of 3 mL
8. Thaw each therapeutic agent you will be using at room temperature. Keep at room temperature for as little time as possible.
9. Treat cells with stimulus.
10. Go through each well and pipette up and down gently without scraping the well with a 1000uL tip to mix each solution.
11. Insert 2 ml of PBS in unused wells and in pockets located between wells to maintain humidity within plate.

12. Incubate the well plates at 37 °C for desired time point.

13. After incubation, remove the contents of each well using a serological pipette into their own respective labeled 15 mL conical tube. Try to avoid scratching the bottom of the well. Each condition you have should have one 15mL conical tube. If conducting duplicates/triplicates for one condition, be sure to still place contents into different 15 ml conical tubes.

14. Wash wells with 1 ml of PBS and add it to respective conical tubes.

15. Add 1 mL of 0.25% trypsin-EDTA to each well and incubate at 37 °C for 3 minutes.

16. Add 1 mL of media to each well to neutralize it.

17. Collect the 2 mL from each well and add that to their same respective 15mL conical tube as in step 13.

18. Wash once again with 1 ml of PBS and add it to respective conical tube.

19. Centrifuge the 15 mL conical tubes for 7 minutes at 1700 rpm.

20. Very slowly aspirate each tube by using a glass pipette. Keep the glass pipette as far from the pellet as possible, while slowly tilting the conical tube until the media is aspirated.

21. Prepare 1X Annexin V Binding buffer by diluting the stock Annexin V 10X Binding Buffer with DEPC H2O. Usually a 10mL aliquot will be prepared by adding 1 mL of Annexin V 10X binding buffer with 9ml of DEPC H2O. Adjust the aliquot size for the number of samples you have.
22. Resuspend the pellets in the 15 mL conical tubes in 1x Annexin V Binding Buffer. If the condition will be having 4 flow tubes (unstained, 2 single stains and 1 double-stain), resuspend the pellet in 800uL of 1x Annexin V Binding Buffer. If the condition will be have 2 flow tubes (unstain and double-stain), resuspend in 400uL of 1x Annexin V binding buffer.

23. Transfer 200uL of each suspension to their respective flow tubes.

24. DONE IN DARK - Then add 5 µL of anti-Annexin V- APC conjugated Antibody to each flow tube and/or 10 µL of PI as appropriate for each flow tube.

25. STILL IN DARK - Once the appropriate stains are added to each flow tube, incubate the flow tubes on ice for 15 minutes. The samples should remain on ice for the remainder of the experiment. Also place the remaining 1X Annexin V binding buffer on ice for the next step.

26. After the 15-minute incubation, add another 200 µL of the cold 1X Annexin V binding buffer to every flow tube.

27. The samples NEED to be run on the LSR flow cytometer shortly after antibody incubation. If you wait more than 30-45 minutes after you complete the staining you WILL see a noticeable difference in your results and will mostly have to repeat the experiment.

28. When running your samples on the LSR, first use your untreated and unstained cells to find your cells. Run the LSR in exponential mode. You want the Y-axis to be PE and the X-axis to be APC. You want to collect 20,000 events for your controls and for your experiment samples. You want to position the cells as close to the lower left corner as possible without cutting
off populations. Then go through your dual stained positive control to make sure everything looks good.

29. Analyze the results using FlowJo. Use your PI only and Annexin V only controls to set your quadrants. You can then see the amounts of apoptosis (top and bottom right hand quadrants).

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC Annexin V</td>
<td>Fisher</td>
<td>550475</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>Fisher</td>
<td>556463</td>
</tr>
<tr>
<td>Annexin V Binding Buffer</td>
<td>Fisher</td>
<td>556454</td>
</tr>
<tr>
<td>6 Well Plates</td>
<td>USA Scientific</td>
<td>T1006</td>
</tr>
</tbody>
</table>
Appendix B:

**MTS Cell Proliferation Assay Protocol**

1. Isolate cells from a flask and transfer to a 15 mL conical tube.
2. Centrifuge the cells at 1700 RPM for 7 minutes.
3. Resuspend pellet in 5mL of complete media, and count cells using a 5uL aliquot into 95 uL of trypan blue solution.
4. Resuspend the cells so that the concentration is $1 \times 10^5$ cells/mL in 1.5 mL Eppendorf tubes. This will lead to having ~5000 cells per well by plating 50 uL in each well. Depending on number of wells being plated, it may be necessary to have more than 1 Eppendorf tube.
5. Prepare each treatment condition in separate 1.5 mL Eppendorf tubes. When preparing the solution, prepare it so it contains 2X the concentration of the desired final concentration as the final volume in each well will be 100 uL (50 uL of treatment, 50 uL of cells). Ex. Desired concentration is 1200 nM, prepare solution in 2400 nM concentration.
6. After each tube is prepared, label a 96 Well Flat Bottom Plate. Make sure each of your treatments is at least done in triplicate. Add the 50 uL of cells (~5000) to wells, followed by 50 uL of treatment media. An example of a well plate layout is below.
<table>
<thead>
<tr>
<th>MTS A375 cells</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td>Blank</td>
<td>Untreated</td>
<td>MLN4924 1200 nM</td>
<td>IFN-α 1X10^4 U/ml</td>
<td>Vem 1µM</td>
<td>MLN+IFN</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td>Blank</td>
<td>Untreated</td>
<td>MLN4924 1200 nM</td>
<td>IFN-α 1X10^4 U/ml</td>
<td>Vem 1µM</td>
<td>MLN+IFN</td>
</tr>
<tr>
<td><strong>Trial 3</strong></td>
<td>Blank</td>
<td>Untreated</td>
<td>MLN4924 1200 nM</td>
<td>IFN-α 1X10^4 U/ml</td>
<td>Vem 1µM</td>
<td>MLN+IFN</td>
</tr>
</tbody>
</table>

7. A 100 µL pipette should be used and a new tip should be used for each well.

8. For blanks plate 100 µL of media alone. After each treatment is plated, fill the remaining wells (if any) with 100 µL of PBS.

9. Incubate the plate at 37 °C for 72 hours.

10. After the 72-hour incubation, add 20 µL of 5.0 mg/mL MTS to each well (except PBS wells). **Everything involving MTS should be done in the dark.**

11. Incubate the well plate at 37 °C for 0.5-2.0 hours, taking absorbance measurements every 30 minutes in the plate reader at 490 nm.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg MTS</td>
<td>Sigma Aldrich</td>
<td>M5655-100MG</td>
</tr>
<tr>
<td>96 Well Flat Bottom Plate</td>
<td>BioExpress</td>
<td>12-565-226</td>
</tr>
</tbody>
</table>
References:


